10/019,052

Mozaic™ Combinatorial Screening Technology

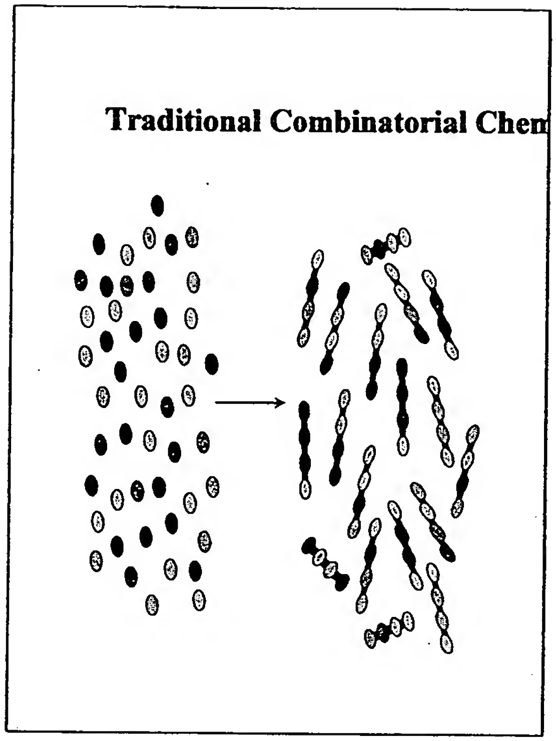
a simple, quick and efficient combinatorial screening system, utilizing unique characteristics of fluid membrane mosaics, to construct receptors capable of binding to cell-surface ligands

The methodology described here is termed 'Moziaic' and is a simple, quick and efficient combinatorial screening system, utilizing unique characteristics of fluid membrane mosaics, to construct receptors capable of binding to cell-surface ligands

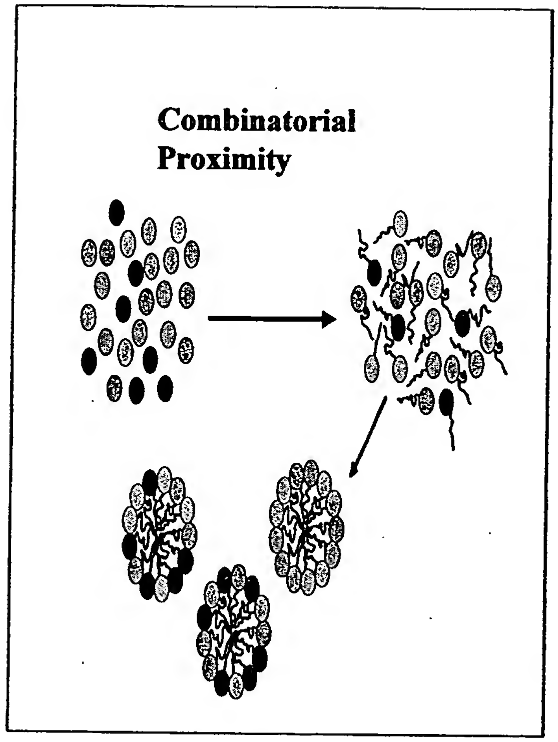
Mozaic™ Combinatorial Screening Technology

- Technology for the discovery of new structures capable of stimulating or down-regulating cells.
- Used to develop
  - new therapeutic entities;
  - targeting ligands; or
  - diagnostic reagents

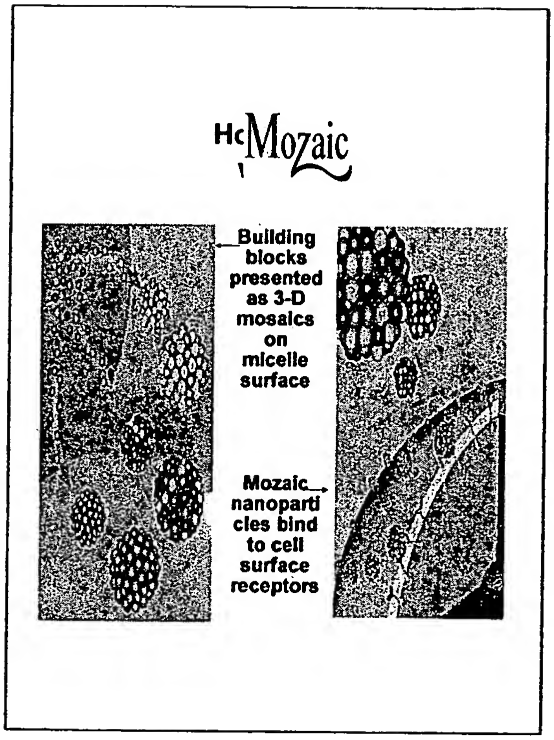
Proxima's unique Mozaic Discovery Screening technology enables one to generate rapidly novel molecular structures which can be used as targeting ligands for drug delivery, diagnostic reagents or therapeutic drug molecules.



To understand the value of the technology, it is helpful to contrast it with conventional combinatorial chemistry. Combinatorial chemistry takes a set of building blocks and links them together in all different sequence permutations, prior to testing each variant in a high-throughput screen. The linkage process is costly, time-consuming, yields small amounts of material, and introduces geometrical constraints in the molecules which often prevents them from adopting the conformations required for optimal binding.



Mozaic uses a 'combinatorial proximity' approach in which the building blocks are brought together without chemistry, thus avoiding geometrical constraints. Each building block is first converted to an amphiphile by attaching it to a lipid tail. These amphiphiles, when dispersed in aqueous phase, spontaneously associate to form micelles whose surface is covered in all directions by the building blocks in a dynamic fluid mosaic array. The building blocks are brought closely together in all different possible configurations, and form structures which can bind to biological receptors. A library of different micelles (or 'probes') can be prepared very easily by selecting different combinations of building blocks from the starting pool.



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Mozaic micelles are ideal agents for stimulation of cells since they can present multiple copies of the same epitope to the cell at the same time. One micelle, therefore, can bind to several receptor molecules concurrently and provide a trigger similar to that seen in cell-to-cell communication. Small molecules in solution are unlikely to be able to do this.

Screening studies can easily be carried out, testing the ability of probes with different combinations of headgroups to modifiy the behaviour of cells in culture.

Structure of Amphiphiles (Pre-combinants)

Amino acids:

Lipophilic	alanine
Hydroxylic	serine
Amide	glutamine
Negative	glutamate
Positive	arginine, histidine, lysine
Aromatic	tyrosine, tryptophan, phenylalanine

Each building block has the general structure shown here, with a constant spacer and lipid tail, and variable headgroups. In this presentation, the technology has been exemplified using amino acids as the building blocks. Because of the flexibility of the building blocks when presented on the micelle surface as amphiphile headgroups, a number of the amino acids having very similar structures to each other are redundant. Consequently it is possible to construct libraries containing all the functionalities of amino acids using just ten out of the whole range of twenty natural amino acids used in proteins in Nature, thereby considerably reducing the screening workload.

Although amino acids have been used here to exemplify the principles of Mozaic, it is very important to stress that the technique is by no means limited to the use of amino acids. Any small molecules can act as a building blocks - steroids, nucleotides, alkaloids, heterocyclics, metallo-organics, and this property of Mozaic distinguishes it clearly from the technologies which rely on genetic mutation to produce combinatorial libraries.

Advantages of Mozaic

- Large amounts of material for testing (no chemistry)
- Many structures on surface of each probe (up to 1,000)

therefore

- Number of test groups is small
- Can conduct screens in cells and whole animals

Because, in Mozaic, large quantities of different micelles can be rapidly prepared, each presenting a large number of different geometric conformations on the same surface, it is possible to test a large number of structures using a library which consists of just a handful of members. This makes the system more amenable to conducting screens not just in simplified high-throughput assays, but also in more complex biological models involving whole cells or even whole animals. Examples of such models are shown later.

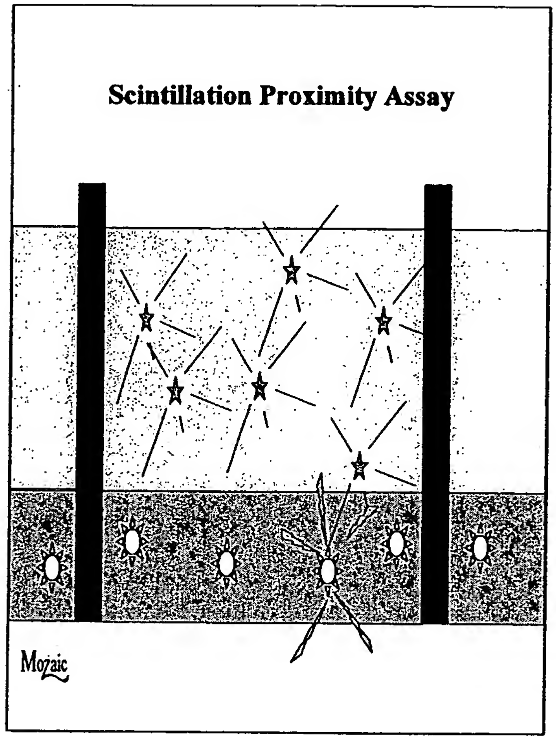
Models for Implementation

1. Protein interactions solid-phase binding,
enzyme inhibition,
stimulation of enzyme
activity

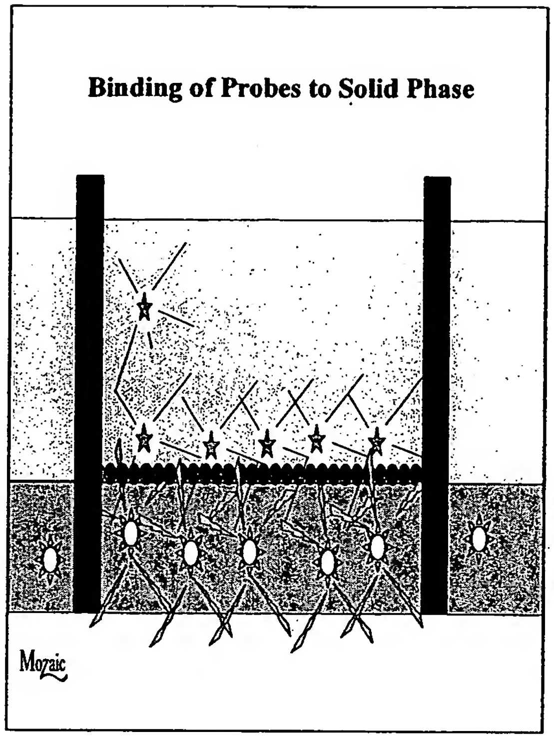
2. Cell-based studies change in growth,
differentiation,
secreted factors,
receptor expression

3. *In vivo* screen look for influence on
outcome
in disease model

The data that follows has been generated using three different assay systems, one involving a solid-phase binding assay for measuring protein interactions (scintillation proximity assay), one using *in vitro* cell culture, and one in which a screen in whole live animals has been conducted.



In the scintillation proximity assay, scintillant is embedded in the base of a microplate, so that when a solution of tritium-labelled micelles is dispensed into the well, only those very close to the bottom of the well can be detected.



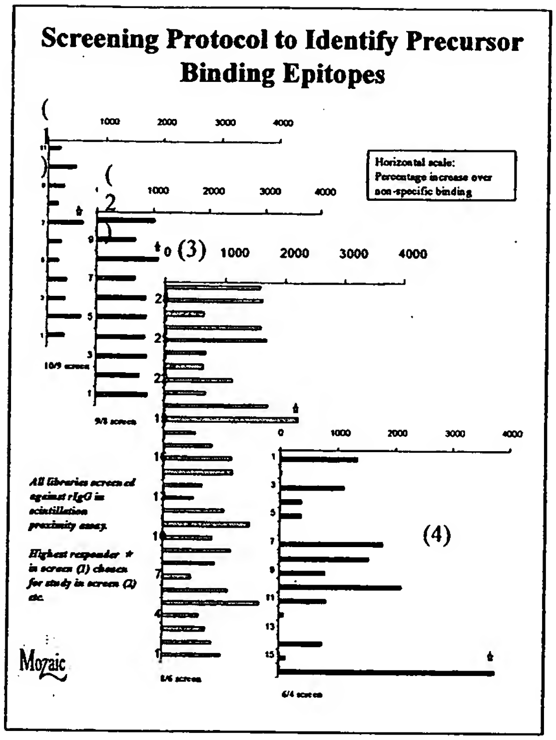
When the bottom of the plate is coated with a protein of interest, the scintillation proximity principle can be exploited to detect binding of micelles to the protein.

Combinatorial Possibilities

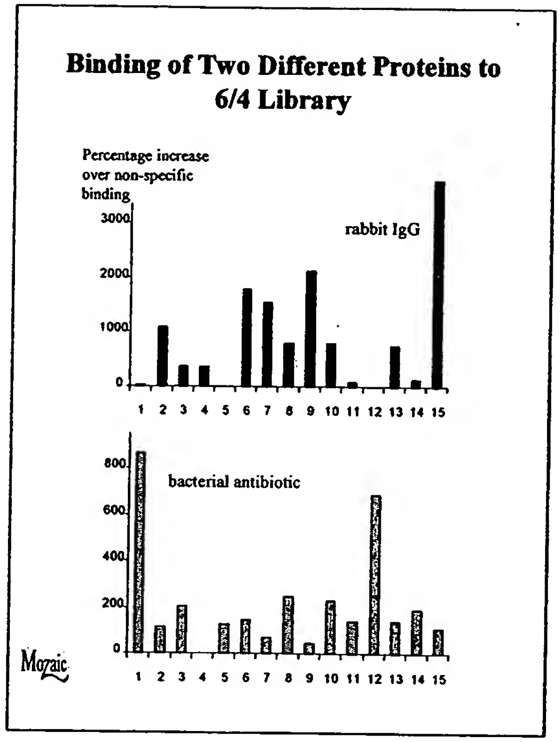
Library name	10/1	10/2	10/3	10/4	10/5	10/6	10/7	10/8	10/9	10/10
No. of members of library	10	10	10	10	10	10	10	10	10	10
No. of members per probe	1	2	3	4	5	6	7	8	9	10
No. of different probes	10	45	120	210	252	210	120	45	10	1
Library name	9/1	9/2	9/3	9/4	9/5	9/6	9/7	9/8	9/9	
No. of members of library	9	9	9	9	9	9	9	9	9	
No. of members per probe	1	2	3	4	5	6	7	8	9	
No. of different probes	9	36	84	126	126	84	36	9	1	
Library name	8/1	8/2	8/3	8/4	8/5	8/6	8/7	8/8		
No. of members of library	8	8	8	8	8	8	8	8		
No. of members per probe	1	2	3	4	5	6	7	8		
No. of different probes	8	28	56	70	56	28	8	1		
Library name	7/1	7/2	7/3	7/4	7/5	7/6	7/7			
No. of members of library	7	7	7	7	7	7	7			
No. of members per probe	1	2	3	4	5	6	7			
No. of different probes	7	21	35	35	21	7	1			
Library name	6/1	6/2	6/3	6/4	6/4	6/6				
No. of members of library	6	6	6	6	6	6				
No. of members per probe	1	2	3	4	5	6				
No. of different probes	6	15	20	15	6	1				

In our experience, micelles with high binding activity only need to contain three or four different building blocks. However, even with a small pool of just ten building blocks this would require over 200 groups to be tested in parallel, which may present problems when running complex cell-culture based assays. An alternative strategy is to start with a screen of ten groups, in which each group contains a different combination of nine building blocks (top row, right hand side). The most positive member of this group gives nine building blocks which which can be tested in nine groups eight at a time (second row), and so on downwards. Building on an early lead in this way, a small number of screens, each refining the results of the preceding screen, can rapidly give a candidate combination of building blocks with high activity, as shown in the following panel.

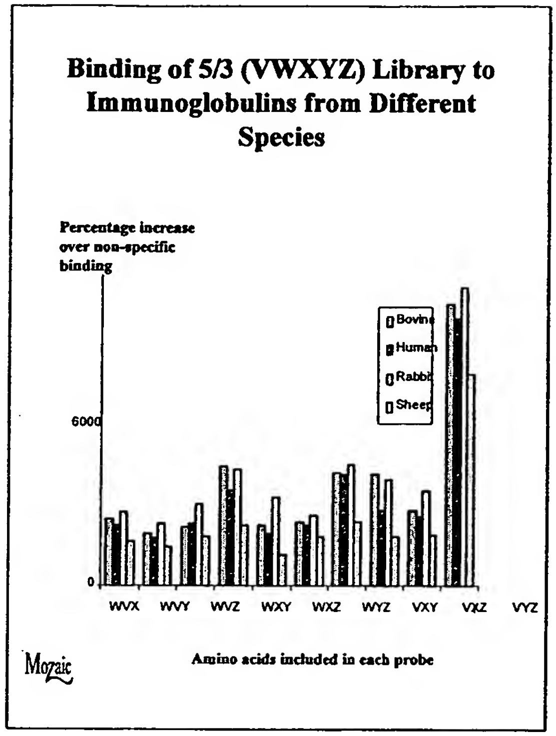
This ‘top-down’ strategy is exemplified in the next panels. An alternative ‘bottom-up’ approach is discussed later on.



Here, four screens have been used in succession to identify a combination of building blocks capable of binding to rabbit IgG. As the screens proceed, progressively higher levels of binding are seen.



As is to be expected, a different binding profile is observed for each different protein. Here, the 6/4 library which was constructed as part of the rIgG programme was run against a bacterial peptide antibiotic. Probes giving positive binding are different in each case.



Immunoglobulins from different species, however, all show similar profiles.

Studies with Lysozyme

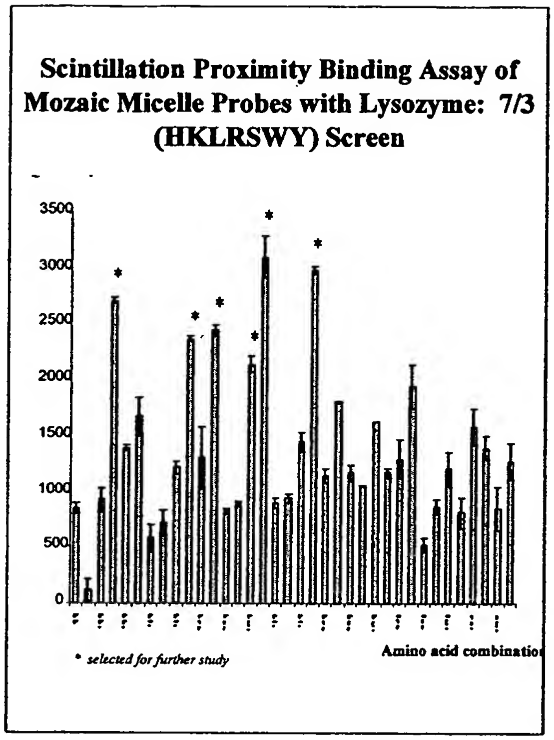
Use of Amino Acid and Sugar Constructs in Combination

In order to develop further the capabilities of the Mozaic technology, as series of studies with lysozyme has been initiated, with the following aims:

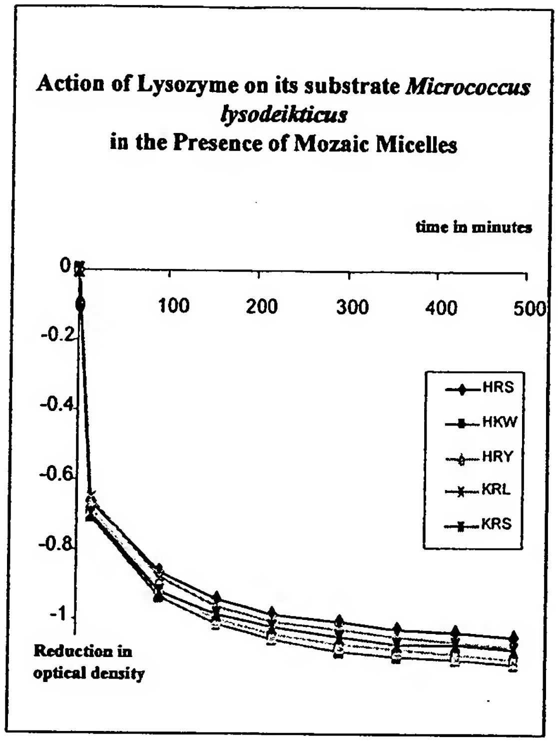
1. To identify structures capable of binding to enzymes, using lysozyme as a model.

2. To study the specificity of binding of the structures identified, in order to demonstrate the feasibility of diagnostic applications of Mozaic.

3. To determine the ability of Mozaic structures to inhibit the activity of lysozyme, in order to demonstrate the feasibility of using Mozaic to create enzyme inhibitors for therapeutic purposes.

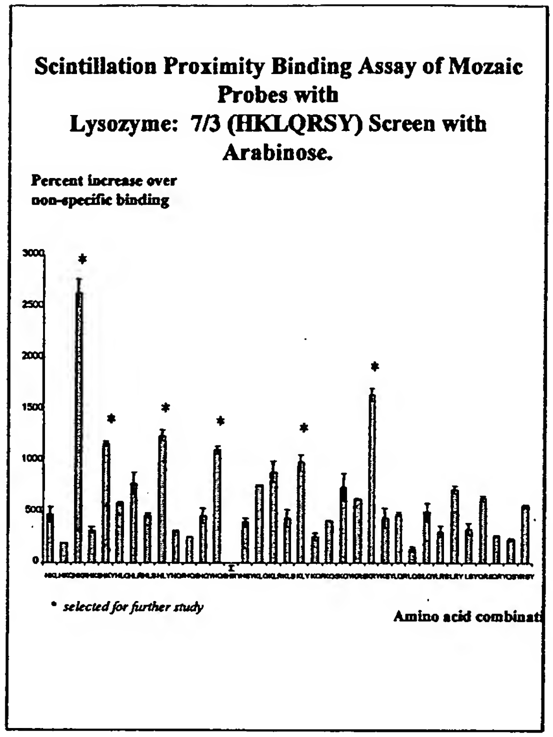


After conducting a number of preliminary screens, a pool of seven (H, K, L, R, S, W and Y) combined in a single probe was shown to display a high degree of binding to lysozyme. This pool was used to construct a library of micelle probes, each containing a different combination of three amino acids. Screening of these probes in the scintillation proximity assay against lysozyme gave the result shown above. Micelles containing six different combinations (the highest binders, shown marked with asterisks) were selected for further study. These were: HRS, HKW, HRY, HWY, KRL and KRS.

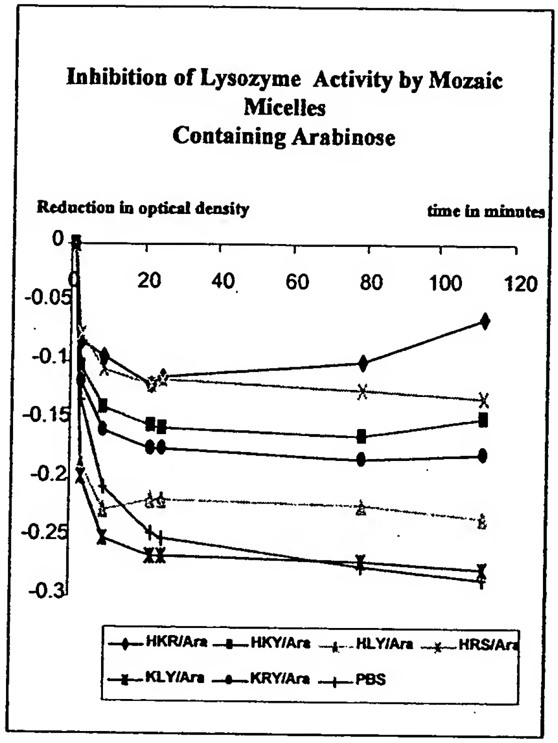


The enzyme activity of lysozyme can be measured by observing its lytic action on a suspension of freeze-dried *Micrococcus lysodeikticus* organisms. The suspension is highly turbid in its original form, scattering light strongly, and registering a high optical density throughout the whole range of visible wavelengths in a spectrophotometer. Upon the action of lysozyme, the suspension is converted to an optically clear solution, with a markedly lower optical density. Experiments conducted in which the reaction is allowed to proceed in the presence of the six high-binding probes identified in the amino acid screen show that no difference can be seen between any of the probes, and that, in spite of their ability to bind to lysozyme, access of the enzyme to the substrate is not blocked.

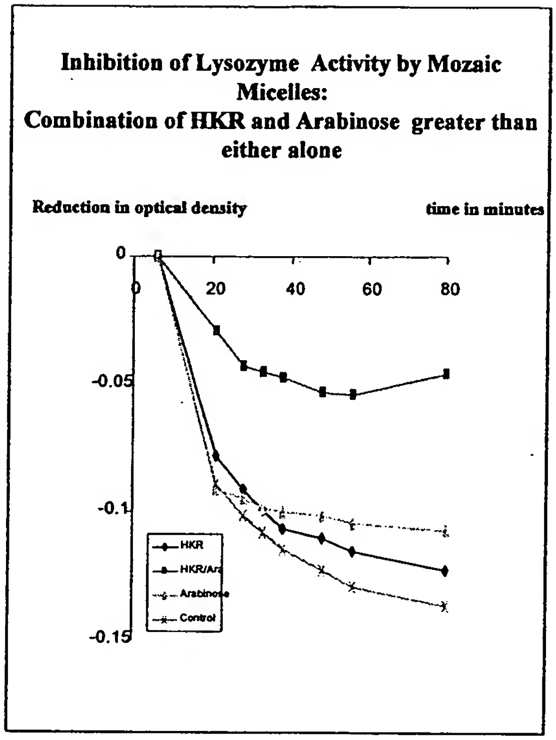
Since the action of lysozyme is to cleave polysaccharide chains on the surface wall of micro-organisms, it was speculated that micelles which contain sugar residues inj addition to amino acids might be more effective in inhibiting enzyme activity, since these would be more likely to bind to the active site of the enzyme. Acccordingly, screens were conducted in which six different sugars were incorporated separately into amino acid-containing micelles, to determine whether any of these might enhance the binding to lysozyme, compared with lysozyme alone.



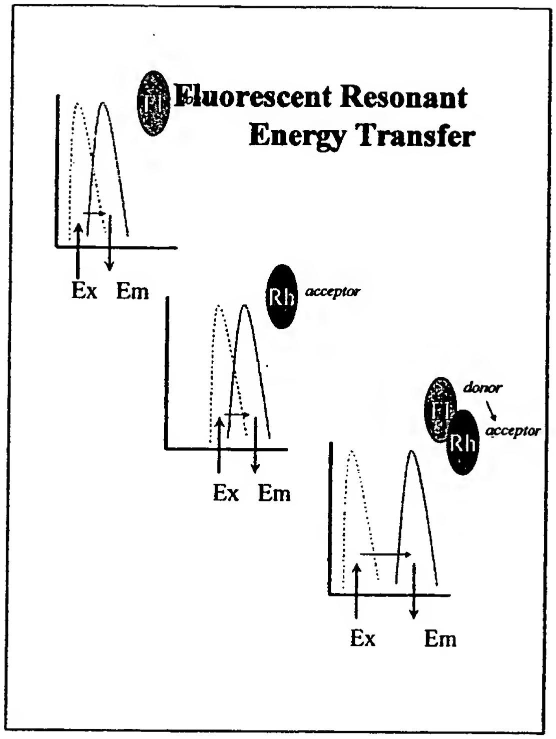
A number of the sugars did indeed appear to enhance binding of probes to lysozyme, and one probe containing HKLQRSY in conjunction with arabinose was selected for further study. A library was constructed containing all possible combinations of these seven amino acids, three at a time, with arabinose present throughout, and a scintillation proximity screen against lysozyme gave the results shown above. Again, the six strongest binders (marked with an asterisk) were selected for further study.



The enzyme reaction was conducted as before in the presence or absence of each of the six highest binders of the arabinose-containing micelles from the studies conducted above. Prior to adding the substrate, the probes were incubated with lysozyme for half an hour. No significant differences in turbidity were seen among the six probes prior to addition of substrate, but differences in clarity achieved were noted after the substrate was added. Whereas one probe showed no difference compared with the control, others appeared to inhibit the reaction to varying degrees. The strongest inhibition observed was with HKR/arabinose.

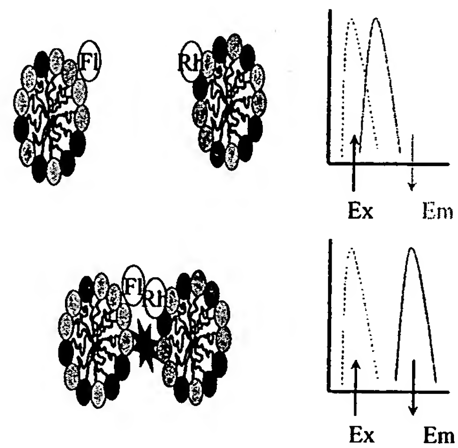


In a further experiment, very little, if any, inhibition by HKR or arabinose alone at the concentrations tested was observable, while the combination of HKR and arabinose in the same micelle continued partially to inhibit the activity of lysozyme.



Fluorescent Resonant Energy Transfer (FRET) can also be used to identify interactions by monitoring the aggregation of Mozaic micelles brought about by binding to proteins. FRET measures the non-radiative transfer of energy between donor and acceptor fluorophores which occurs only when the two molecules are in very close proximity to each other.

Fluorescent Enhancement upon Binding of Protein to Micelles

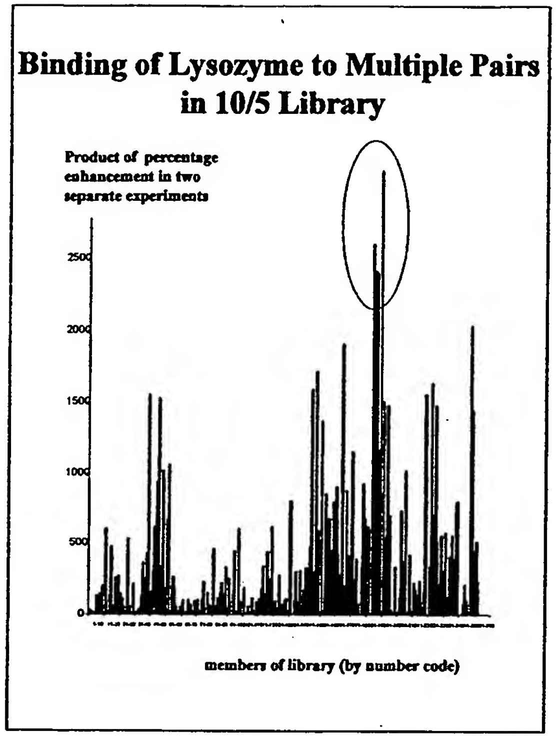


If the donor (fluorescein) and acceptor (rhodamine) are located on different micelles, than an increase in light emitted at the rhodamine wavelength is seen on when a protein binds to both micelles and brings them into close proximity.

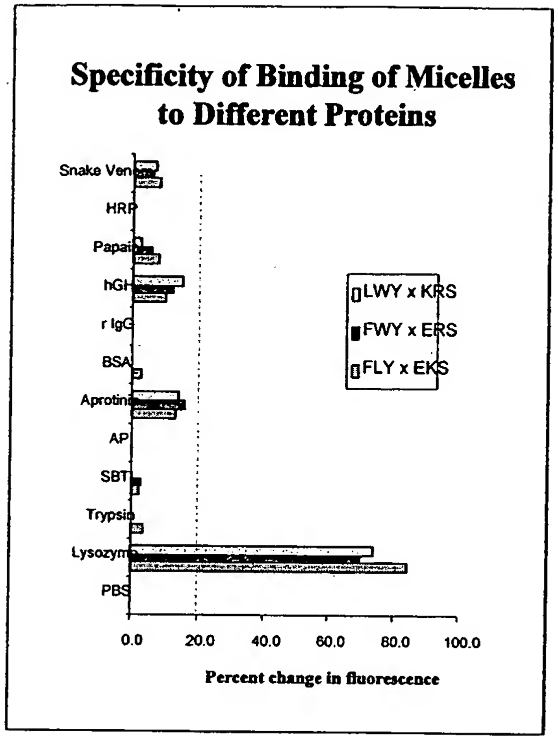
airs Involved in Multiple Binding Analy of 10/5 Library

	Fluorescein	*Rhodamine*
1	ABCDE	FGHIJ
2	ABCDF	EGHIJ
3	ABCDG	EFHIJ.....
	CDEFG	ABHIJ
	CDEFH	ABGIJ
	CDEFI	ABGHJ.....
250	EFHIJ	ABCDG
251	EGHIJ	ABCDF
252	FGHIJ	ABCDE

The screen is conducted in such a way that each of the 252 different combinations of fluorescein micelle is paired off with the rhodamine micelle which contains the complementary set of five out of ten procombinants which are absent from the fluorescein micelle. This is done to maximise the chance that the two micelles present will bind to different sites on the protein, and thus be able to bind to the same protein at the same time.

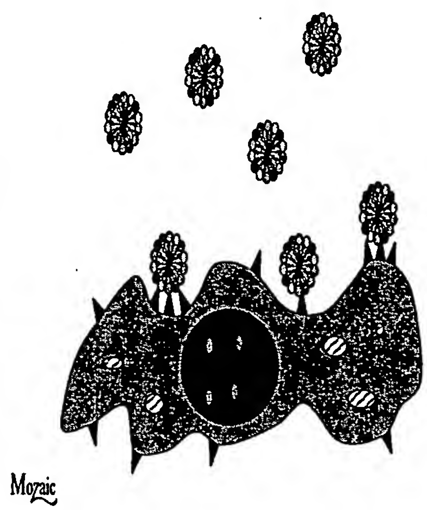


Conduct of the screen, using lysozyme as a model, shows clusters of pairs with high binding activity, as is to be expected, since micelles adjacent to each other in the library will differ by only one or two amino acids, and will thus contain several identical combinations of amino acids which will give rise to identical precursor epitope binding sites.



Further screens can be conducted to dissect out the combinations of amino acids responsible for activity in the high bindin micelle pairs. As a result, in the lysozyme model, three pairs of micelles have been identified, each with three amino acids, which show specificity for lysozyme, while a wide range of other proteins all give response below the threshold level.

Stimulation of Cells by Probes



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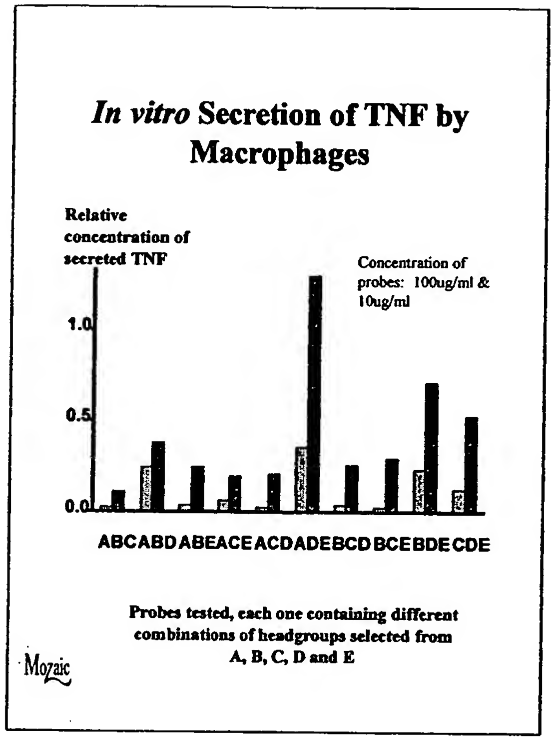
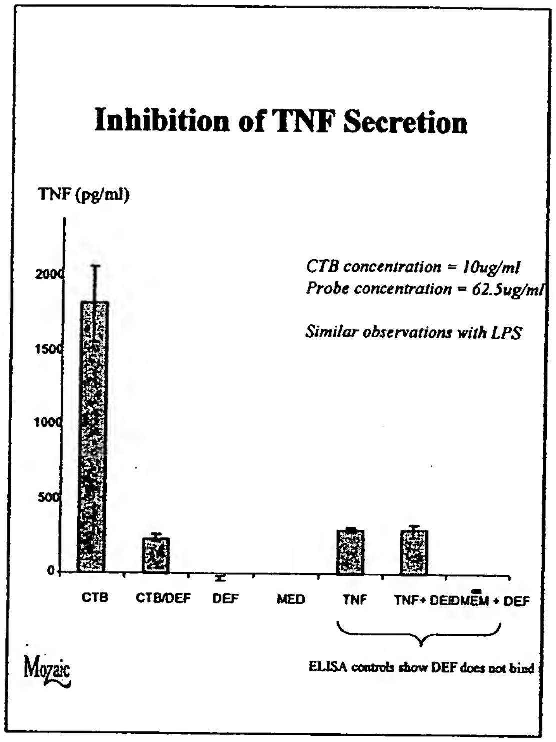
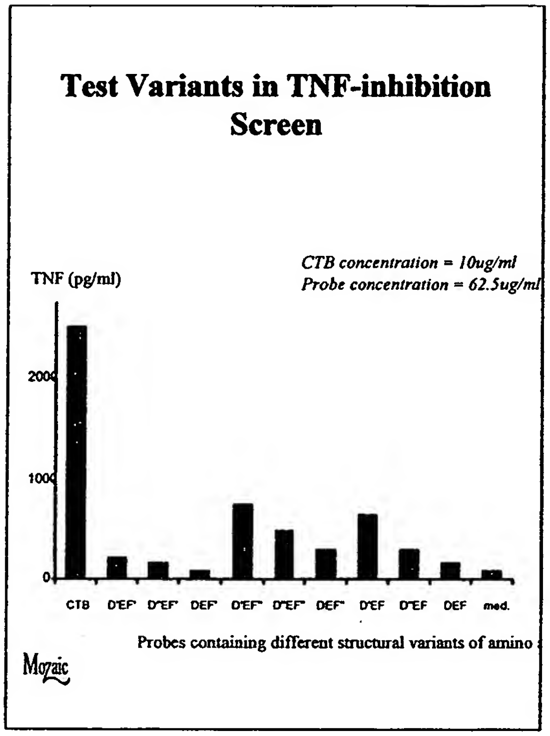


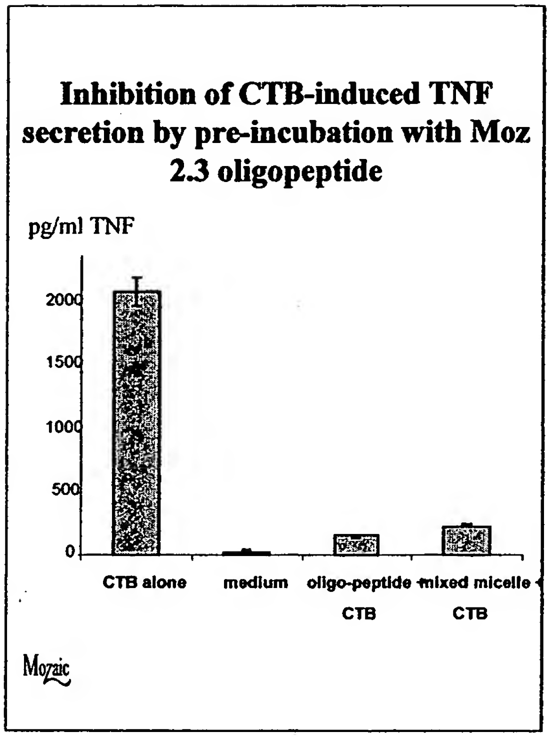
A portion of a screen conducted in J774 macrophages is shown here, where it can be seen that different micelles behave very differently in regard to their ability to stimulate secretion of TNF by these cells. The activity of the micelle ADE is equivalent, on a wt/wt basis, to that of cholera toxin B fragment - a very potent stimulator of TNF.



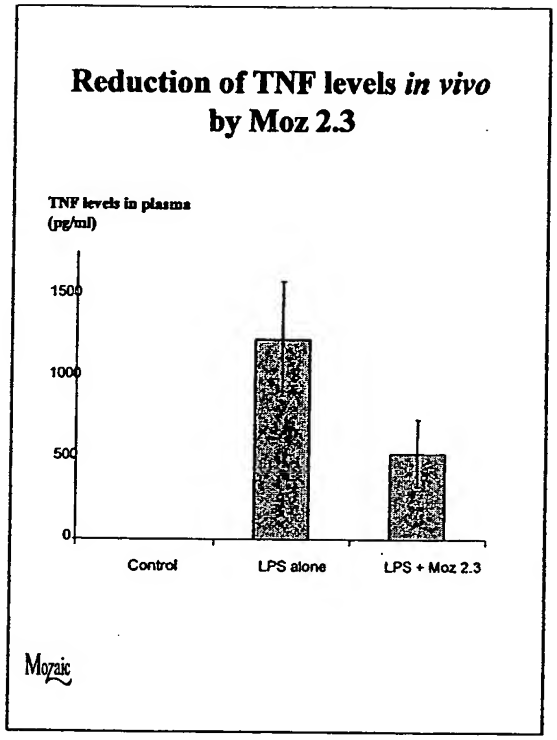
Additional studies with J774 cells have led to identification of another probe (DEF) which can down-regulate secretion of TNF stimulated by either cholera toxin B fragment (second column), or bacterial lipopolysaccharide (data not shown here).



Further screens looking at structural variants of the building blocks D and F have identified a combination which can inhibit TNF secretion altogether (DEF', column 4).



A linear peptide construct has been synthesised which contains the same amino acid building blocks as before, linked together in a chain, and then attached to the surface of a micelle. This possesses the same level of inhibitory activity as the mixed micelle containing the amino acids separated from each other.



Moz 2.3 is also able to exert its effect *in vivo*. Rats treated with 1mg of lipopolysaccharide intra-peritoneally gave a high level of TNF in the bloodstream 1.5 hours after administration. Dosing with Moz 2.3 half an hour before LPS reduced the TNF level by over 50%.

Studies with Cancer Cells

1 Binding

2 Growth inhibition

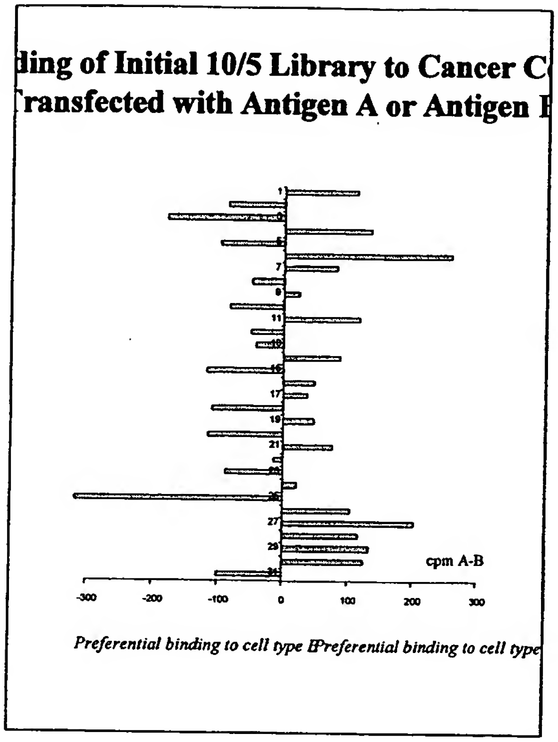
The Mozaic system can be employed to identify structures which will bind to specifically to cancer cells, and could be used to target cytotoxic drugs in carrier vesicles to these cells in vivo.

Alternatively, screens can be conducted to determine combinations of amino acids which are capable of killing and/or altering the growth rate of cancer cells in a tumour-specific fashion.

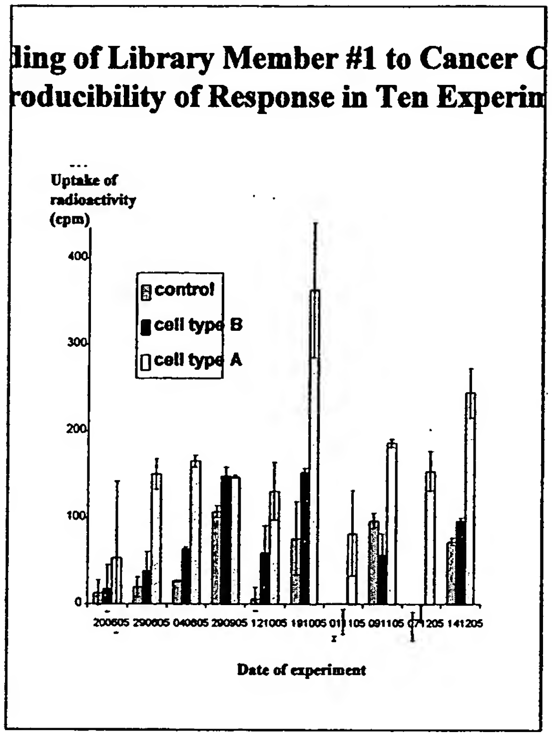
truction of Initial 10/5 Library Compri All Possible Triplets (32 members)

ABCEH	ABC	4	ABH	2	ABE	3	ACH	3	ACE	3	AEH	4	BCH	3
	BCE	2	BEH	2	CEH	4								
ABCDJ	ABC	4	ABD	3	ABJ	4	ACD	2	ACJ	2	ADJ	2	BCD	4
	BCJ	2	BDJ	3	CDJ	3								
ABCDI	ABC	4	ABI	2	ABD	3	ACI	3	ACD	2	ADI	3	BCI	2
	BCD	4	BDI	2	CDI	4								
ABHIJ	ABH	2	ABI	2	ABJ	4	AHI	4	AHJ	3	AIJ	5	BHI	2
	BHJ	2	BIJ	3	HIJ	2								
AGHIJ	AGH	2	AGI	2	AGJ	2	AHI	4	AHJ	3	AIJ	5	GHI	2
	GHJ	2	GIJ	3	HIJ	2								
ABCFG	ABC	4	ABF	2	ABG	3	ACF	2	ACG	2	AFG	2	BCF	3
	BCG	4	BFG	4	CFG	3								
ABFGJ	ABF	2	ABG	3	ABJ	4	AFG	2	AFJ	2	AGJ	2	BFG	4
	BFJ	2	BGJ	3	FGJ	3								
ABEIJ	ABE	3	AFI	3	ABJ	4	AEI	3	AEJ	4	AIJ	5	BEI	2
	BEJ	2	BIJ	3	EIJ	3								
AEFIJ	AEF	2	AEI	4	AEJ	4	AFI	3	AFJ	2	AIJ	5	EFI	3
	EFJ	2	EIJ	3	FIJ	2								
ADEIJ	ADE	4	ADI	3	ADJ	2	AEI	4	AEJ	4	AIJ	5	DEI	3
	DEJ	3	DIJ	2	EIJ	3								
ACFHI	ACF	2	ACH	3	ACI	3	AFH	3	AFI	3	AHI	3	CFH	2
	CFI	2	CHI	2	FHI	4								
ACEHJ	ACE	3	ACJ	2	ACH	3	AEJ	4	AEH	4	AHJ	3	CEJ	3
	CEH	4	CHJ	2	EHJ	2								
ADEFH	ADE	4	ADF	2	ADH	3	AEF	2	AEH	4	AFH	3	DEF	2
	DEH	4	DFH	4	EFH	3								
ADEGH	ADE	4	ADG	2	ADH	3	AEG	3	AEH	4	AGH	2	DEG	4
	DEH	4	DGH	3	EGH	2								
ABDEG	ADE	4	ADG	2	ABD	3	AEG	3	ABE	3	ABG	3	DEG	4
	BDE	3	BDG	4	BEG	2								
BDFHJ	BFH	3	FBD	3	BFJ	2	BHJ	2	FHJ	2	DFH	4	BDH	2
	DFJ	3	BDJ	3	DHJ	3								
EFGHI	EFG	2	EFH	3	EFI	3	EGH	2	EGI	3	EHI	3	FGH	3
	FGI	2	FHI	4	GHI	2								
DFGHJ	DFJ	3	DGJ	2	DHJ	3	DFG	2	DFH	4	DGH	3	FGJ	3
	FHJ	2	GHJ	2	FGH	3								
CEFGJ	CEJ	3	CFJ	2	CGJ	2	CEF	2	CEG	3	CFG	3	EFJ	2
	EGJ	2	FGJ	3	EFG	2								
BCDEF	BCD	4	BCE	2	BCF	3	BDE	3	BDF	3	BEF	2	CDE	4
	CDF	2	CEF	2	DEF	2								
CDEHJ	DEH	4	CDE	4	DEJ	3	CDH	3	DHJ	3	CDJ	3	CEH	4
	EHJ	2	CEJ	3	CHJ	2								
CDEHI	CDE	4	CDH	3	CDI	4	CEH	4	CEI	3	CHI	2	DEH	4
	DEI	3	DHI	2	EHI	3								

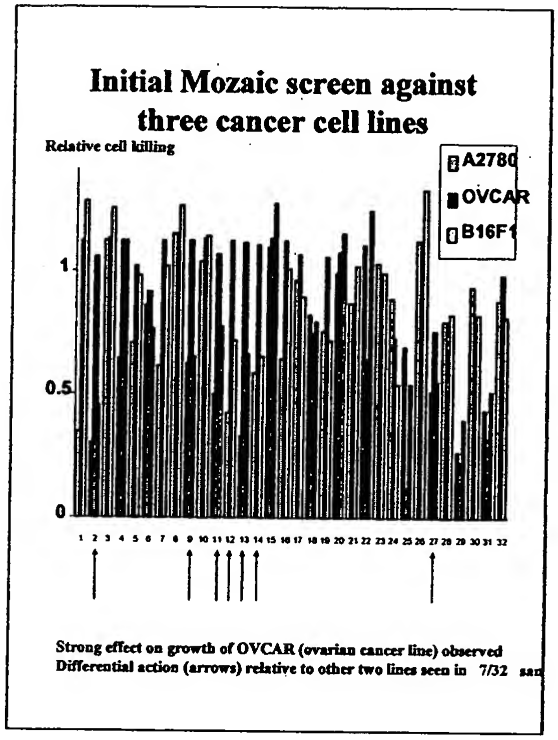
In order to construct a library which contained all possible combinations of ten functional amino acids in as small a number of different groups as possilbe, the assumption was made that the binding epitpoes capable of bringing about the desired reaction would contain no more than three amino acids. A library was then constructed, each member of which contained a different combination of five of the ten amino acids, in such a way that each combination of three amino acids which can be made up from the starting pool of ten is represented two or more times. Each combination of five amino acids gives rise to ten different combinations of three The above table shows the thirty two members of the library, and the total number of times each combination of three is represented. There are 120 combinations of three in total.



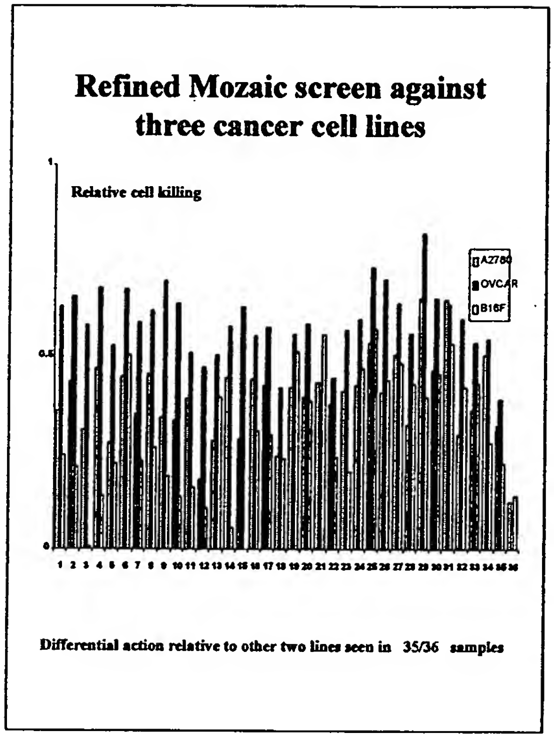
In one set of experiments, binding of the library by melanoma cells transfected by one or other of two specific antigens (but otherwise identical) was measured using the scintillation proximity assay.



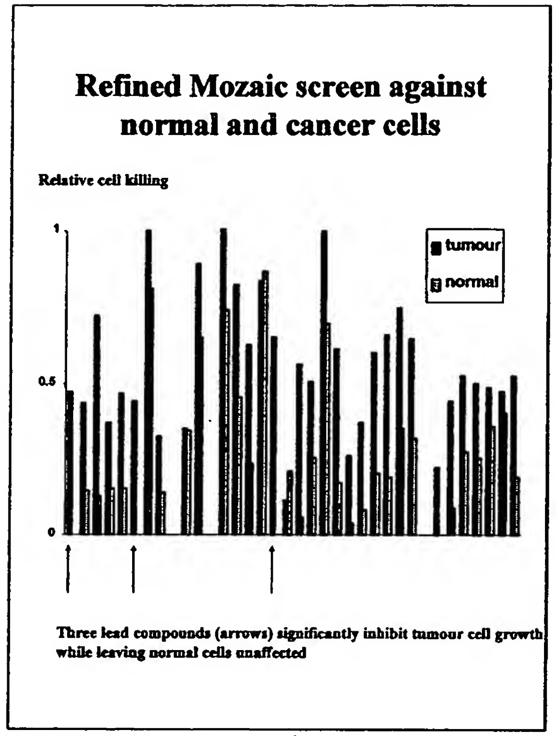
The experiment was repeated ten times to demonstrate the reproducibility of binding to micelle #1.



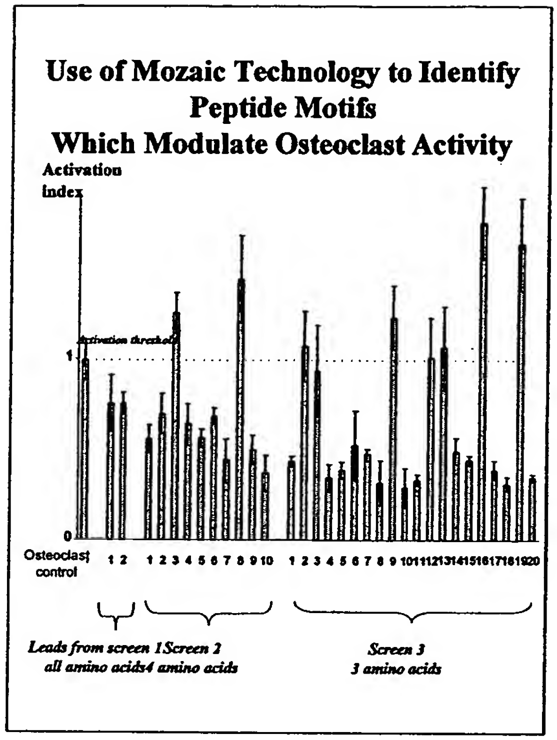
In a separate study, the same 10/5 library was screened against three tumour cell lines – two ovarian cancers and one melanoma – to determine whether amino acid combinations could be identified which had a cytostatic or cytotoxic effect on the tumour cells. In the first screen, seven of the combinations were identified which had a significantly greater effect on OVCAR-3 than on the other two cell lines. A second, more refined library was then constructed, based on the combination contained within these seven test samples alone.



Screening of the second library showed that combinations of four amino acids had been identified which again had a greater effect on OVCAR-3 than on the other two. In this second iteration, the number of samples showing a significant differential increased from 7 out of 32, to 35 out of 36.



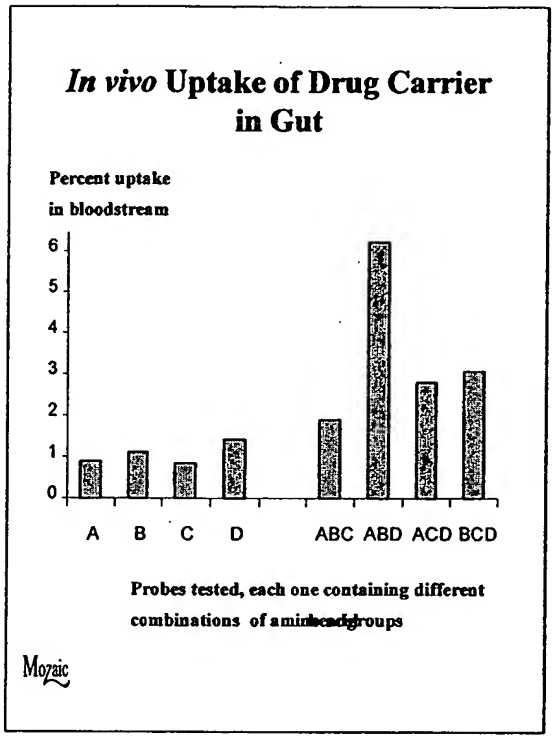
The same library was tested to determine whether the cytotoxic effects seen were specific to cancer cells, or whether normal cells would also be killed off. In three samples in the library, growth inhibition was indeed observed for the ovarian cancer cell line under conditions where normal endometrial cells were unaffected.



Several iterations of a screen were carried out to identify combinations capable of inhibiting osteoclast activity in RANKL-stimulated cultured cells. After each iteration, the level of inhibition of osteoclast activation increased. The most active combinations of three amino acids identified in screen 3 are currently being incorporated into Lexicon constructs, to produce potential calcitonin mimetics with oral activity.

Mozaic Screen
Conducted *In Vivo*

Mozaic can also be used in screens involving *in vivo* test systems.



Here, radio-labelled micelles were administered orally, and blood samples taken, over a 2 hour period, to find a combination of building blocks which could assist the transport of the micelles from the intestinal lumen, across the gut wall, and into the bloodstream. A small portion of the total screen, involving thirty different groups altogether, is shown here. Micelles consisting of just single building blocks (A, B, C & D)can be considered as negative controls. However, when certain of these building blocks are put together, an increase in uptake of an order of magnitude can be observed (ABD). This combination could be employed in Lexicon peptide constructs to make them orally available.

Mozaic Applications

Target ***Activity***
Application

Immune cells secrete cytokines
vaccines
inhibit cytokines rh. arthritis

Cancer cells enhance uptake
drug delivery
induce apoptosis tumour
reduction

Stem cells differentiation
neural disorders
proliferation diabetes

Intestine/BBB transcytosis
oral delivery

CNS disease

Microbes neutralisation
viral bacterial

No other combinatorial screening technology is able to work with such a wide range of biological test systems, or conduct screens in models so close to the clinical end-point. Because of the unique way in which building blocks are presented in Mozaic, structures can be generated and developed to therapeutic application which would never be detected by other techniques.

The list of potential applications for the Mozaic/Lexicon technologies is very long. A number of the projects cited above are already being conducted by Proxima in-house.